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A homolog of *Blade-On-Petiole 1* and *2* (*BOP1/2*) controls internode length and homeotic changes of the barley inflorescence

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Summary:

Loss of function mutation of a gene, homologous to known transcriptional regulators involved in setting organ boundaries of *Arabidopsis thaliana*, causes homeotic changes in the barley inflorescence.

FOOTNOTES:

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ABSTRACT

Inflorescence architecture in small grain cereals has a direct effect on yield and is an important selection target in breeding for yield improvement. We analyzed the recessive mutation *laxatum-a* (*lax-a*) in barley, which causes pleiotropic changes in spike development resulting in: (i) extended rachis internodes conferring a more relaxed (lax) inflorescence, (ii) broadened base of the lemma awns, (iii) thinner grains that are largely exposed due to reduced marginal-growth of the palea and lemma, (iv) and homeotic conversion of lodicules into two stamenoid structures. Map-based-cloning enforced by mapping-by-sequencing of the mutant *lax-a* locus enabled the identification of a homolog of *BLADE-ON-PETIOLE1* and *2* (*BOP1* and *2*) as the causal gene. Interestingly, the recently identified barley *UNICULME4 (HvCUL4)* gene is also a *BOP1/2* homolog and has been shown to regulate tillering and leaf sheath development. While the Arabidopsis *BOP1* and *BOP2* genes act redundantly, the barley genes contribute independent effects in specifying developmental growth of vegetative and reproductive organs, respectively. Analysis of natural genetic diversity revealed strikingly different haplotype diversity for the two paralogous barley genes indicating different modes of selection acting either directly on both genes or on additional factors located in strong linkage disequilibrium (LD).

INTRODUCTION

The inflorescence is the most prominent part of small grain cereal plants, producing the carbohydrate rich grains that are harvested for food, feed and fibre. However our understanding of the genetic factors that regulate inflorescence architecture remains limited. What is clear is that the appearance and shape of the inflorescence has been under constant visual selection since early domestication and is still ongoing in modern plant breeding due to the impact of inflorescence architecture on crop yield. For instance in barley, strong selection has been exerted on spontaneously occurring alleles of *BRITTLE RACHIS 1 and 2 (btr1/btr2)* that prevent dehiscence of the rachis at maturity (Pourkheirandish et al., 2015), *SIX-ROWED SPIKE 1* (*VRS1*) that determines whether the inflorescence exhibits two or six rows of grain (Komatsuda et al., 2007), and *NUDUM* (*nud)* that controls whether the grain is hulled or hulless (Taketa et al., 2008). Ultimately, knowing all of the genes that control cereal inflorescence architecture will provide targets for understanding and exploiting natural or induced genetic diversity towards improving both yield potential and end-use characteristics.

The barley inflorescence or 'spike', forms an unbranched main rachis carrying triplets of sessile single-floreted spikelets, inserted at opposing sides of subsequent rachis nodes, that leads to the characteristic two-rowed (lateral spikelets infertile) or six-rowed (all three spikelets at a node are fertile) phenotype of domesticated barley. Each fertile barley floret is composed of a central carpel surrounded by a whorl of three stamens and two lodicules, and enclosed by two leaf-like structures, the palea and the generally long-awned lemma (Kellogg, 2001). The genes controlling barley inflorescence architecture and development have only been revealed for a few characters. Major genes that control row type (*VRS1*, Komatsuda et al., 2007; *INTERMEDIUM-C* (*INT-C*), Ramsay et al., 2011; *VRS4*, Koppolu et al., 2013), convert awns into an extra floret (*HOODED* (*K*), Muller et al., 1995), adherence of the hull to the caryopsis (*NUD*, Taketa et al., 2008), swelling of lodicules conferring open/closed flowering (cleisto-/chasmogamy) (CLY1, Nair et al., 2010), elongation of awns and pistil morphology (*LKS2*, Yuo et al., 2012), suppression of bracts (*TRD1*, Whipple et al., 2010; Houston et al., 2012), spike density *(ZEO1, Houston et al., 2013),* spike branching (COM2, Poursarebani et al., 2015) and brittleness of the rachis (*BTR1/BTR2*, Pourkheirandish et al., 2015) were recently cloned. A large number of additional morphological mutants that influence barley inflorescence development (Forster et al., 2007) have also been described, and the underlying genes need to be identified to reach a more complete understanding of the regulatory pathways controlling barley spike architecture and development (Forster et al., 2007).

The recessive *laxatum-a* (*lax-a*) mutant exhibits a pleiotropically altered spike architecture characterized by (i) extended rachis internodes conferring a lax inflorescence architecture,

(ii) a broadened base of the lemma awns, (iii) thin and exposed grains due to a impaired palea development, (iv) as well as a homeotic conversion of the lodicules into two additional stamen which are smaller and have only two locules instead of four in the regular three stamen (Larsson, 1985; Laurie et al., 1996).

Here we describe the identification and characterization of the gene that is disrupted in the *lax-a.8* mutant. Located in a region of severely suppressed recombination frequency, the gene was identified using an innovative mapping- and cloning-by-sequencing approach. It encodes a homologue of the *Arabidopsis thaliana* putative transcriptional co-activator genes *BLADE-ON-PETIOLE 1* and *2* (*BOP1/2*). Similarities in the morphological defects of *BOP-like* mutants indicated a partially conserved function between Arabidopsis and barley.

RESULTS

The *laxatum-a* phenotype

The original *laxatum-a.8* (*lax-a.8*) mutant was obtained by fast neutron mutagenesis of the cultivar 'Bonus' (Franckowiak, 2010). For genetic analysis the mutation was introgressed previously by repeated backcrossing into the cultivar 'Bowman' to produce the nearly-isogenic line (NIL) BW457 (Druka et al., 2011). The term 'laxatum' refers to its relaxed ('lax') spike phenotype which is manifested by an average of 15% increase of rachis internode length in BW457 if compared to wild-type Bowman (Figure S1). The unique pleiotropic characteristics of *lax-a* set it apart from other laxatum-type mutants: its awns have a broad base, and its lodicules are homeotically converted into stamen of smaller size and comprising only two instead of four locules (Figure 1). The thin and angular grains of *lax-a* mutants are exposed at spike maturity. This is the result of incomplete covering of grains by outer lemma and inner palea due to impaired development of their marginal regions. Interestingly, *lax-a* mutants can open flowers without impetus force of lodicules (Figure 1).

Mapping-by-sequencing of pooled recombinant plants revealed a candidate gene for the *lax-a* mutation

We performed conventional high-resolution genetic mapping followed by exome capture sequencing of selected recombinant plants to identify a candidate gene for the *lax-a* locus. The nearly isogenic line (NIL) BW457 carried a 38.5 cM introgression interval on chromosome 5H harboring the mutant locus (Druka et al., 2011). Initial low resolution mapping allocated the gene to a 1.5 cM interval. The *lax-a* phenotype co-segregated with a cluster of four markers spanning the genetic centromere of chromosome 5H (Figure S2). Mapping was extended to a population of 1,970 F_2 plants which delimited an interval of 0.2 cM on the long arm of chromosome 5H (Figure S2). The rather small genetic interval related to a large physical interval and was predicted to contain a minimum of 200 genes based on the analysis of conserved synteny to sequenced model grass genomes (Mayer et al., 2011). Thus the gene was located in a region with significantly reduced recombination frequency – a characteristic, typical in barley, for loci at close proximity to the genetic centromere (IBSC, 2012). All flanking markers were anchored by sequence comparison to the physical map of barley (IBSC, 2012) but a single physical map contig could not be identified (Table 1). Eight recombination events characterized the high resolution genetic mapping interval of 0.2 cM carrying the gene *lax-a*. The respective eight recombinant F2 plants could be grouped into five different classes of recombinant haplotype / phenotype combinations that we called lax-1 to lax-5 (Figure 2A). DNAs of individuals belonging to the same class were then pooled for target enrichment shotgun re-sequencing (Mascher et al, 2013a). More than 30 million

properly paired sequence reads were obtained per sample (Table S1) and single nucleotide polymorphism (SNP) frequencies were determined between the pooled, sequence-enriched DNA samples and wild-type Bowman and visualized in the context of the physical / genetic map of barley (Figure 2B and S3). In all five pools an easily traceable shift in overall SNP frequency could be observed at the expected recombination breakpoints previously determined by low-density genotyping (Figure 2B). Due to the highly reduced recombination frequency at the genetic centromeres of barley chromosomes, the genetic resolution of barley physical map order was low in this region, and the correct linear order of a large part of the allocated physical map information in this area of the barley chromosomes was rather uncertain. Thus, the immediate identification of a highly resolved and limited physical target interval carrying the *lax-a* locus was not achieved. Consequently, the analysis was modified by categorizing SNPs from the pool sequencing as co-dominant genotype scores according the percentage of reads with mutant alleles mapped at the variant site. SNP positions with a frequency of less than 20% mutant reads were scored as wild-type (wt/wt) genotype, with more than 80% mutant reads as homozygote mutant (mt/mt) and between 20 to 80% as heterozygote genotype. The relaxed thresholds in genotype calls were implemented due to the risk of false positive read mappings, e.g. by misidentification of the respective multiplexing index (Kircher et al., 2012), or to avoid the risk of bias in SNP frequencies introduced by less read coverage of targets in one of the pools. This allowed us to filter for SNPs in targets that cosegregate with the respective phenotypic characteristic of each pool independently from the status of anchoring on the physical map (Figure 2). In total, 15 WGS contigs containing targets with SNPs following the required pattern were identified (Table S2). Nine high confidence genes (Table S4) were identified by sequence comparison to the barley gene set (IBSC, 2012).

Since the *lax-a* mutation was obtained by fast neutron mutagenesis (Franckowiak, 2010), which frequently introduces partial or complete gene deletions (Li and Zhang, 2002), we surveyed for targets of the enrichment assay that were not covered by sequence reads in the pools of recombinant plants with mutant phenotype. If the gene was represented in the target enrichment design and it was deleted (at least partially) due to mutation, no sequence read coverage would be expected for mutant phenotype pools whereas wild-type phenotype pools of recombinant plants should exhibit normal (average) read coverage for the respective target enrichment regions. Due to the above mentioned risk of false positive read mappings, a maximum read coverage threshold of 2-fold was accepted for pools with mutant phenotype (lax-3 and lax-4). Furthermore, a minimum read coverage of 5-fold required at similar target sites for wild-type phenotypic pools (lax-1, lax-2 and lax-5). This analysis revealed 12 additional WGS contigs with putative candidate targets (Table S3) which were analysed for genes by sequence comparison to the barley gene set (IBSC, 2012). One gene,

MLOC_61451.6, was detected that was not covered by sequence reads in the mutant phenotype recombinant pools lax-3 and lax-4, indicating its likely deletion in the *lax-a* mutant (Table S5). This gene therefore represented a strong candidate gene conferring the *lax-a* phenotype.

Only a few of the 27 WGS contigs identified by the two parallel approaches were already integrated into the physical map framework of barley (IBSC, 2012), thus their physical relationship remained unclear at this stage. We considered synteny between the barley and Brachypodium genomes in order to deduce a potential physical order of the genes represented on the respective 27 WGS contigs. Seven out of the 10 putatively orthologous gene models were allocated to a small physical interval (144 Kbp) in Brachypodium. The Brachypodium ortholog of the potentially deleted barley gene was located in the center of this syntenic block (Figure 2D).

The gene *HvLAX-A* resides in a 450 kbp deletion in the mutant BW457

As the WGS contig bearing the candidate gene was not linked to the barley physical map, the candidate gene MLOC_61451.6 was used to PCR screen a barley BAC library and four clones (HVVMRXALLeA0046E04, HVVMRXALLeA0122D17, HVVMRXALLeA0209O12, HVVMRXALLeA0379H14), all belonging to the same BAC contig (Finger Printed Contig, FPC) FPcontig_2862 of the physical map (Ariyadasa et al., 2014), were identified. A minimum tiling path of overlapping non-redundant BAC clones, spanning the complete FPcontig_2862 was shotgun sequenced and *de novo* assembled yielding 2.3 Mbp of unique sequence (Table S6). WGS survey-sequencing data of genotypes 'Bowman' and mutant NIL BW457 were mapped against the newly generated BAC contig reference sequence. This analysis predicted a deletion of 436/554 Kbp (min/max) in the mutant genotype BW457 in the center of FPcontig_2862 (Figure S4). Based on previously existing gene annotation (IBSC, 2012), four genes were identified on the entire sequenced BAC contig, however, only MLOC_61451.6 was affected by the deletion (Figure S4, Table 2, Table S7). The candidate gene consists of two exons and a single intron (Figure 3A). PCR amplification from cDNA of cv. Bowman was performed to determine splice site positions (data not shown). The predicted CDS of MLOC_61451.6 erroneously contained six additional base pairs that are actually part of the intron.

Mutant analysis confirmed the identification of the gene *HvLAX-A*

In order to test whether the deletion of candidate gene MLOC_61451.6 confers the *lax-a* mutant phenotype, the gene was re-sequenced in a series of 29 independent *lax-a* alleles in mutant accessions obtained from the Nordic Genome Resource Center (http://www.nordgen.org) (Table 3). In fourteen genotypes the candidate gene could not be

amplified indicating its complete deletion. The failure of PCR in these cases cannot be attributed to highly polymorphic primer binding sites due to a putatively diverse haplotype of the genotypic background of these mutants, since the gene could be amplified from independent alleles induced in the same genetic background of cultivar 'Bonus', 'Foma' or 'Kristina' (Table 3). Fourteen accessions carried point mutations, which in seven cases resulted in the formation of premature stop codons, six resulted in non-synonymous amino acid substitutions and one resulted in an altered splicing site. One mutant carried two mutations: a 2 bp deletion and one single base deletion in close proximity causing a single amino acid deletion and one amino acid exchange (Figure 3B, Table 3). Among the Bowman near isogenic lines (Druka et al., 2011), an additional accession BW458 was described to exhibit the *lax-a* mutant phenotype. In this accession the first part of exon1 could not be amplified by PCR, again indicating a partial gene deletion (Figure 3). All analyzed mutant accessions expressed the characteristic *lax-a* phenotype.

In addition to re-sequencing the existing allelic series of *lax-a* mutants, a population of 7,979 EMS-mutagenized plants (Gottwald et al., 2009) was screened by Targeting Induced Local Lesions In Genomes (TILLING). Eighteen mutations leading to non-synonymous amino-acid changes were identified (Table S8). A single mutation, C127T (L43F, mutant 8476), revealed the typical *lax-a* phenotype showing five anthers, extended rachis internode length, broadened base of the lemma awn and uncovered seeds (Figure S5). The respective M3 family was segregating for the mutation and only the two homozygous mutant M3 plants showed the *lax-a* phenotype, consistent with the recessive nature of the mutated gene. The same mutation was shared by an allele (*lax-a.278*, NGB116503) of the above described lines from the Nordic Genome Resource Center (Table 3). A test for allelism was performed between the mutant line used for cloning (BW457), the TILLING mutant 8476 and BW458, respectively. All F1 plants exhibited the typical *lax-a* phenotypic syndrome (Figure S5) thus confirming the allelic status and further supporting that MLOC_61451.6 is the functional gene underlying the *lax-a* phenotype in respective barley mutants. Consequently, the gene was named *HvLAX-A.*

HvLAX-A* is a homolog to Arabidopsis *BLADE-ON-PETIOLE 1* and *2

Sequence comparison of *HvLAX-A* revealed homology to the *Arabidopsis thaliana* genes *BLADE-ON-PETIOLE 1 and 2* (*BOP1 and BOP2*), which together control leaf morphogenesis and floral organ abscission (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). Both genes belong to a small gene family in Arabidopsis and gene members carry conserved BTB/POZ (BROAD COMPLEX, TRAMTRACK, and BRICK À BRACK/POXVIRUSES and ZINC FINGER) and ANK (ANKYRIN repeats) domains. The gene family in Arabidopsis includes the genes *BOP1* and *BOP2* as well as the four plant defense related

NONEXPRESSOR OF PR GENES1-like (*NPR1-like*) (Hepworth et al., 2005). A combined analysis of these *Arabidopsis* genes and their respective barley homologs revealed a conserved phylogeny of *BOP* and *NPR1-like* genes in barley (Figure S6). *HvLAX-A* and its closest barley paralog, AK360734, represented the putative orthologs of *Arabidopsis thaliana* genes *BOP1 and BOP2.* However, the level of sequence conservation between barley and Arabidopsis did not allow us to determine absolutely the orthology relationship between the members of both gene pairs.

HvLAX-A and its paralogous gene affect different aspects of barley plant architecture

In Arabidopsis, *BOP1* and *BOP2* have partially redundant functions (Hepworth et al., 2005; Norberg et al., 2005). To test the hypothesis that in barley, mutations in the two *BOP-like* genes could result in similar phenotypic alterations, we screened the barley TILLING population to identify functional mutants for *AK360734*. A series of 25 non-synonymous and 21 synonymous mutations, one premature stop codon (9425_1) and one splice site mutation (13391_1) were identified (Table S9). Mutants affected by non-synonymous amino acid exchanges did not show any obvious phenotypic effect. The two mutants affected either by a premature stop codon or a splice site mutation, respectively, exhibited a 'ligule-less' phenotype with irregular outgrowth of auricles (Figure S7 D-F) and produced only less than three tillers (Figure S7 B-C). M3 families segregated for the mutation with perfect linkage of homozygous mutant genotype and the described phenotypic alterations. Phenotypic effects were severe (stunted growth, strong curling of the shoot and leaves), and only 6 out of 15 homozygous mutant plants grew to maturity (4 plants of M2 family 9425_1; 1 plant of M2 family 13391_1). None of the plants showed any characteristic *lax-a* changes. The observed phenotypic syndrome resembled exactly a pattern previously reported for the barley mutant *uniculme4* (*cul4*, Tavakol et al., 2015). Indeed, when completing our TILLING analysis of *AK360734*, the same gene was reported as the underlying factor of the *uniculme4* phenotype (Tavakol et al., 2015). Our analyses therefore provided independent confirmation of these findings.

Natural variation of _BOP-like_ genes in barley

HvLAX-A and its paralog *HvCUL4* are involved in regulating two major agronomically relevant traits, tiller number and spike morphology. Thus natural alleles of both genes could have been under selection during barley domestication, adaptation or more recent breeding. To test this, we investigated the natural diversity for both barley *BOP-like* genes *HvLAX-A* and *HvCUL4*. The ORF of both genes was amplified in a set of 83 wild (*Hordeum spontaneum*) and 222 domesticated barley lines consisting of landraces and improved cultivars (Table S10). The majority of accessions (205, 67%) carried one major haplotype for

HvLAX-A and the remaining genotypes belonged to nine minor haplotypes specified by 11 polymorphic sites (S) consisting of nine synonymous and two non-synonymous changes (Figure 4, Table 4). For *HvCUL4* a significantly higher number of polymorphic sites and a more diverse haplotype structure was revealed (Figure 4). The 48 polymorphic sites were represented by 38 synonymous and 10 non-synonymous SNPs (Table 4). In addition, some accessions carried a 3 bp (domesticated material) or 6 bp (wild material) insertion within the second exon of *HvCUL4.* The *HvLAX-A* and *HvCUL4* haplotype diversity (hd) was decreased by about 11.8% (from hd=0.644 to hd=0.0781) and 18.7% (hd=0.9412 to hd=0.765) in domesticated versus wild barley, respectively. Nucleotide diversity (π) was reduced as well for *HvLAX-A* (from π = 0.0009 to π = 0.0005) but in contrast was slightly increased for *HvCUL4 (from 0.00456 to 00493)* between wild and domesticated material (Table 4). The Tajima D test (Tajima, 1989) for neutrality of DNA polymorphisms was performed to test if the observed changes in polymorphisms were likely caused by a non-random shift or by natural selection. None of the calculated Tajima's D values reached significance ($p<0.1$), thus, the observed changes in sequence diversity were considered to be unlikely a function of selection of the two analyzed genes.

Phylogenetic analysis of the *BOP*-like genes

A sequence database screen in a previous study revealed the existence of two to three *BOP-like* genes in most plant species (Khan et al., 2014). Sequence conservation between Arabidopsis *AtBOP1* and *AtBOP2* and barley *HvLAX-A* and *HvCUL4* did not allow defining their orthology relationship. Therefore, we extended the phylogenetic analysis to a larger number of angiosperm, fern and moss species to better reveal the evolutionary relationships between gene family members, and support hypotheses of putative functional orthology. A maximum likelihood-based phylogenetic tree was constructed based on protein sequences. This analysis showed that, despite the existence of multiple BOP-homologs in mosses and ferns, the gene family members in higher land plants were unlikely to have evolved directly from these evolutionary distant copies (Figure S8). The analyses did not allow us to determine which non-angiosperm *BOP-like* gene was conveyed to younger angiosperms. Recently, based on the higher sequence conservation of dicot *BOP-like* genes it was proposed that they may have originated from more recent duplication events than the monocot genes. This may go some way towards explaining the functional redundancy of the Arabidopsis genes (Tavakol et al., 2015). The *BOP-like* genes in the Poaceae showed a unique pattern of diversification compared to the other analyzed, exclusively dicot, *BOP* families. While the *HvCUL4*-containing clade was closely related to all BOP-like members of dicot plant families, the *HvLAX-A*-containing clade was more distinct. One may conclude that *HvCUL4* is orthologous to either *BOP1* or *2*. *HvLAX-A* (and the respective orthologs of other

Poaceae), however, was either a direct but more diversified BOP1/2 ortholog or originated as a Poaceae-specific gene duplication followed by neo- / sub-functionalization leading to higher sequence diversity.

DISCUSSION

Mutation of *HvLAX-A* causes pleiotropic phenotypic aberrations in the barley inflorescence including an elongated spike rachis, homeotic conversion of lodicules into stamenoid structures, thin and angular grains exposed at spike maturity due to reduced width of palea and lemma and a broadened base of the lemma awn. The functional gene, *HvLAX-A,* resides in the genetic centromere of barley chromosome 5HL, a region characterized by very low recombination frequency. It was identified after deploying an innovative strategy of mapping- and cloning-by-sequencing as a homolog of the transcriptional regulator genes *BLADE-ON-PETIOLE 1* and *2* (*BOP1* and *2*) of *Arabidopsis thaliana*.

HvLAX-A, a key regulatory gene of barley inflorescence architecture

HvLAX-A, showed strong sequence homology to the *AtBOP1/2* genes, which in Arabidopsis are expressed in lateral organ boundaries and regulate redundantly the proximal and distal growth of leafs, the floral transition and organ identity (Ha et al., 2004; Hepworth et al., 2005; McKim et al., 2008). Single loss of function mutants in Arabidopsis showed no or very weak phenotypic effects, whereas double mutants were severely affected in growth (Hepworth et al., 2005; Norberg et al., 2005). Florets of *AtBOP1/2* mutants exhibited a loss of floral organ abscission and changes of symmetry in conjunction with the formation of extra floral organs, with fused organs often appearing on the abaxial site of florets (Hepworth et al., 2005). In barley, *lax-a* florets did not exhibit supernumerary organs, but lodicules were homeotically transformed into stamenoid organs and an ectopic growth of the bases of the lemma awn was observed. The leaf alterations in Arabidopsis *bop1/2* mutants more closely resembled the phenotypic effects in mutants of the second barley *BOP-like* gene, the *UNICULME4* mutant (Tavakol et al., 2015). *HvCUL4* was shown to control the number of tillers, ligule development and proximal-distal leaf patterning. Interestingly, in barley loss-of-function mutation even of a single *BOP-like* gene resulted in the respective phenotypic alterations. Complete loss of *HvLAX-A* did not result in any obvious effect on leaf composition or tillering, and deletion of *HvCUL4* did not impact spike architectural traits. The differences between Arabidopsis and barley may be the result of increasingly specific sub-functionalisation of *BOP1/2* homologs in barley, a hypothesis that will require further testing by, for example, analyzing the phenotypic characteristics of *lax-a/cul4* double mutants.

HvLAX-A and *HvCUL4* are to our knowledge the first *BOP-like* genes characterized in detail in monocot species and as a result the underlying regulatory networks of *BOP-like* genes remain largely unknown. The observed phenotypic similarities between *bop-like* mutants in barley and Arabidopsis could indicate at least partially conserved function. In Arabidopsis, the *BOP1/2* regulatory network has been extensively explored. *BOP1/2* act in organ boundaries and control meristem activity by regulating the expression of *KNOTTED-like* homeodomain (*KNOX*) and *BEL1-like* Homeodomain (*BELL*) gene family members which belong to the three-amino-acid-loop-extension (TALE) class of proteins. In Arabidopsis these genes are important for meristem maintenance and organ differentiation (reviewed in (Hamant and Pautot, 2010; Khan et al., 2014). *AtBOP1/2* promote the expression of the lateral organ boundary (LOB) domain gene *ASYMMETRIC LEAVES2* (*AS2*) which forms a complex with *ASYMMETRIC LEAVES1* (*AS1*) to suppress expression of the *KNOX* genes *BREVIPEDICELLUS* (*BP*), *KNAT2* and *KNAT6* in leaves (Jun et al., 2010). In contrast, the opposite mode of regulation has been reported to control inflorescence stem elongation. The *BP* and *BELL-like* homeodomain gene *PENNYWISE* (*PNY*) function as repressors of *BOP1/2*, *KNAT6* and the *BELL* gene *ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1)*, while *BOP1/2* activates *KNAT6 and ATH1* in a shared pathway (Khan, 2012a; Khan, 2012b; Khan et al., 2014) Recently, it was shown that *BOP1/2* function as negative regulators of the bZIP transcription factor FD, which is a component of the floral transition pathway of *FLOWERING-TIME LOCUS T* (*FT*) and is required for the activation of key floral development regulatory genes *LEAFY* (*LYF*) and *APETALA1* (*AP1*) (Andres et al., 2015). Opposing roles for *BOP1/2* were proposed in later stages of floret development via the direct promotion the expression of *AP1* and *LYF*, thus controlling floral organ patterning (Karim et al., 2009; Xu et al., 2010; Andres et al., 2015).

As the role of *KNOX* genes in plant development is conserved between dicots and monocots (Hay and Tsiantis, 2010) the reported *BOP1/2* dependent *KNOX* gene regulation in Arabidopsis could indicate similar activity in monocots. As an example, mutations in *HvKNOX3* lead to the HOODED phenotype in barley, when an extra floret is formed on the lemma at the transition zone between the leafy part and the awn (Muller et al., 1995). The *lax-a* characteristic broadening at the base of the awn could indicate an incomplete version of such transition and thus point at *HvBOP-like* and *HvKNOX* gene interaction. In rice, a relatively close relative of barley, the transcription factors (TF) of the *JAGGED LATERAL ORGANS* (*JAG*) and *YABBY* TF family have been reported as regulators of *KNOX* gene expression in rice panicles. Both TF classes regulate *KNOX* gene expression in a shared pathway together with *AtBOP1/2* and get repressed in Arabidopsis leafs and bracts (Norberg et al., 2005; Ha et al., 2007; Jun et al., 2010). Mutations in the rice *JAG* homologous gene

OPEN BEAK (*OPB*) / *STAMENLESS 1* (*SL1*) (Horigome et al., 2009; Xiao et al., 2009) cause pleiotropic alterations in panicle structure similar to barley *lax-a* mutants: plants exhibit elongated panicles, reduced growth of the lemma and palea as well as alterations in the number and identity of floral organs. *OPB* acted as suppressor of *KNOX* genes, since loss-of-function of *OPB* results in ectopic expression of rice *KNOX* genes (Horigome et al., 2009). These analogies in rice and barley anatomy and the similarities in the regulatory networks among rice and Arabidopsis, may again imply that *BOP*-gene regulatory pathways are partially conserved between monocot and dicot plants.

The *OsYABBY5* gene *TONGARI-BOUSHI1* (*TOB1*) in rice plays a role in maintenance and organization of floral meristems (Tanaka et al., 2012). Loss-of-function mutants show a reduced number of floral organs and varying growth changes in lemma and palea while ectopic expression of *TOB1* increases the number of all floret organs. These pleiotropic growth changes are correlated with the expression and spatial distribution of the *KNOX* gene *OSH1* (Tanaka et al., 2012) indicating the importance of spatial regulation of *KNOX* gene expression in rice for meristem fate during inflorescence development. Similarly, the pleiotropic spike alterations found in *lax-a* mutants could be the result of spatio-temporal changes in *KNOX* gene expression, suggesting that the barley *BOP-like* gene *HvLAX-A* could fulfill a central function in controlling meristem identity as does *BOP1/2* in Arabidopsis.

In addition to their potential effect on *KNOX* gene regulation, *BOP* genes in barley may also be involved in the regulation of MADS Box TF. In Arabidopsis, *BOP1/2*-regulated expression of *AP1* leads to down regulation of *AGAMOUS-LIKE24* (*AGL24*), a homolog of *AGAMOUS* (*AG*). Ectopic expression of *OsMADS3*, the rice ortholog of *AGAMOUS* (*AG*) (Kyozuka and Shimamoto, 2002) leads to homeotic transformation of lodicules into stamens. The conversion of lodicules into stamens in *lax-a* could therefore potentially be explained by a lack of *AG* down regulation in lodicules. While there is some support for such speculation, further experiments, such as quantitative transcriptional profiling of microdissected immature spike meristems, will be required to conclusively demonstrate the postulated role of *HvLAX-A* and *HvCUL4* in *KNOX* and *MADS* BOX gene regulation in barley.

Natural diversity analysis

Since *HvLAX-A* and *HvCUL4* are regulators of agronomically important traits such as spike morphology and tiller number, we explored whether the sequences of both genes in barley accessions collected from different environmental and geographical origins revealed putative signatures of selection during early or recent barley adaptation or improvement. *HvLAX-A* exhibited a very low level of diversity, even in wild barley accessions. The complete deletion

of *HvLAX-A* is not lethal and most non-synonymous mutations did not induce any visible phenotype in greenhouse grown plants, it was somewhat unexpected to find such a low level of natural sequence diversity. We believe that this was unlikely the result of purifying selection of *HvLAX-A* and was more likely a direct consequence of its location in a low-recombining chromosomal region; as nucleotide diversity is correlated with recombination rate it is generally reduced in the low-recombining genetic centromeres (Begun and Aquadro, 1992). In these regions, extensive linkage disequilibrium (LD) to other, yet to be identified, factors under strong selection could have led to fixation of *HvLAX-A* already in the wild barley genepool.

In contrast, *HvCUL4* showed high nucleotide diversity relative to *HvLAX-A*. The diverse *HvCUL4* haplotype network could not be explained by population structure in the analysed panel (Haseneyer et al., 2010) and could not be attributed to specific regional adaptation concerning latitude / longitude coordinates. *HvCUL4* nucleotide diversity in domesticated barley accessions was slightly increased compared to wild barley, although estimates of Tajima's D in cultivars, landraces and wild barley failed to reach significance. We conclude that differences in nucleotide diversity were most likely caused by random bottleneck effects characteristic for the analyzed population. Extending this analysis to a larger number of accessions as well as to the flanking genomic region would be required to reach more definitive conclusions.

Among the identified polymorphisms in *HvCUL4*, premature stop codons and splice site mutations that would have major negative impacts on protein function were not observed. Although, we did not characterize the accessions for phenotypic variations, we do not expect an overly negative impact of the identified non-synonymous polymorphisms since they were exclusively located outside of the putative functional domains. Even under greenhouse conditions *HvCUL4* TILLING mutants exhibited phenotypic changes with severe negative effects on fitness. Thus, although higher sequence diversity was observed when compared to *HvLAX-A* in our panel of genotypes, it is likely that severely affected alleles would be under strong negative selection in natural populations. Consequently, the molecular variation we observed revealed no indication of an active selection process for either gene during domestication, adaptation and improvement. It seems more likely that the patterns of diversity we observed are simply a product of the contrasting genomic environments in which either gene resides.

Cloning-by-sequencing in barley – does genomic position matter?

Forward genetics has been feasible, but labor-intensive, in barley for several years (Stein, 2005). Based on bulked segregant analysis (BSA) (Michelmore et al., 1991) mutation identification by high-throughput sequencing was recently established in Arabidopsis (for review see (Schneeberger and Weigel, 2011) and was extended to barley by combining the approach with target enrichment re-sequencing (Mascher et al., 2014). Cloning-by-sequencing of barley *MANY NODED DWARF* (*HvMND*) was exceptionally successful, but was largely enabled by the location of the gene in a recombination hot-spot. Here we applied a modified approach to clone the gene *HvLAX-A,* necessitated by its location in a region of highly suppressed recombination. We found it was critical to select highly informative recombinants by high resolution genetic mapping and pool these for BSA sequencing. Two additional features facilitated *HvLAX-A* identification: the target gene was included in the target enrichment assay and the mode of mutagenesis resulted in complete deletion of the functional gene. As the barley genome sequence was largely an unordered draft (IBSC, 2012; Ariyadasa et al., 2014) our initial genetic analysis revealed a large physical region of the chromosome represented by highly fragmented sequence information that required a substantial amount of further work to fully resolve. In the near future, the cloning of genes in the non-recombining part of barley chromosomes will become much more efficient given the impending release of a highly improved physical map-based reference sequence. This is both timely and important since a long history of barley mutant research has resulted in well-characterized and easily accessible mutant collections in managed gene banks (Lundquist, 2009). A large number of mutants chosen to represent the morphological and developmental variation observed in the species have been backcrossed in to a single isogenic background of cv. Bowman that have already been genetically characterized in some detail (Druka et al. 2009). They represent a valuable resource for systematic gene isolation by mapping- and/or cloning-by-sequencing approaches in the immediate future.

Conclusion

In the present study we identified *HvLAX-A*, a homolog of Arabidopsis *BOP1/2* genes, that is involved in inflorescence development in barley. The paralogous gene, *HvCUL4*, independently controls the leaf blade to sheath boundary formation and tillering. *BOP1/2* gene regulatory networks have been extensively explored in Arabidopsis. The identification of paralogous genes in barley revealed both conserved and divergent functions in dicot and monocot plant species. The comparative analysis of the underlying regulatory networks will greatly be facilitated in the future due to the cloning of both *BOP-like* genes of barley.

MATERIALS AND METHODS

Plant material

For genetic mapping, the NIL BW457 (Druka et al., 2011), bearing an introgressed segment with the fast-neutron induced *lax-a.8* mutant allele (Franckowiak, 2010), was backcrossed to the recurrent parent Bowman to generate an F_2 population.

For mutant analysis, additional *laxatum-a* mutant accessions were investigated. Besides the Bowman NIL BW457, used for mapping the *lax-a.8* locus, line BW458 was described to exhibit the five stamen phenotype (Druka et al., 2011). In addition, 29 *lax-a* accessions (Table 3) were available from the Nordic Genetic Resource Center (NordGen, http://www.nordgen.org). All plant material was cultivated under greenhouse conditions (18°C / 16°C day / night temperature). Natural light as well as additional sodium lamps were used for illumination.

Mutant analysis

A TILLING population of 7,979 preexisting EMS-treated plants of cv. Barke (Gottwald et al., 2009) was screened for independent mutant alleles of *HvLAX-A*. Two primer pairs were used to amplify the full ORF (HvLAX_EX1_F/R and HvLAX_Ex2_F/R, Table S11) by a standard PCR with a final heteroduplex step as described earlier (Gottwald et al., 2009). PCR products were digested with DNF-480-3000 dsDNA Cleavage Kit and analyzed using DNF-910-1000T Mutation Discovery 910 Gel Kit on the *AdvanCE*™ FS96 system according to manufacturer's guidelines (Advanced Analytical, Iowa, USA). Identified SNPs were confirmed by Sanger sequencing (see below). All mutants carrying non-synonymous SNPs were addressed for phenotyping. The genomic sequence of *HvLAX-A* of independent *lax-a* accessions was generated by PCR amplification and subsequent Sanger sequencing to identify sequence variations. The Bowman NILs BW457 and BW458 were amplified by PCR with four primer combinations: HvLAX-F/R1, HvLAX_F/R2, HvLAX_F/R3 and HvLAX_F/R4. The *lax-a* mutant accessions from NordGen were analyzed by PCR amplification of primer combinations (HvLAX_F/R_7 to 11; Table S11). Mutant line lax-a.373 (NGB Nr. 116583) was excluded from the analysis because of missing additional stamen phenotype.

Allelism test of independent *lax-a* mutant alleles

The Bowman NIL BW457 was used as female parent and crossed with TILLING mutant 8476 and NIL BW458, respectively. A codominant SNP marker test for heterozygosity was performed to confirm the success of the cross. Since *HvLAX-A* was identified to be located within a large deletion, two flanking polymorphic SNPs, located on FPcontig_2862 outside of

586 the deletion, were used for genotyping the cross of BW457 and BW458 (Table S14). Two
587 primer combinations (Table S11) were used to amplify these polymorphic SNPs (Table S14)
588 and all analyzed F1 plants proofed to be heterozygous for both markers. All sixteen F1 plants
589 displayed a *lax-a* characteristic phenotype (Figure S5 R-V). For the second cross, between
590 BW457 and the TILLING mutant 8476, only the SNPs on Bowman_contig_129575 could be
591 utilized for co-dominant genotyping (Table S14). To generate a second independent
592 confirmation, the first exon was amplified (HvLAX_F/R4, Table S11) in all F1 plants as
593 dominant marker and the obtained fragment was sequenced. All fragments carried the
594 homozygous mutant allele (T) at position 127 bp, transferred from the male parent TILLING
595 Mutant 8476. In total six F1 plants were analyzed for the cross between BW457 and TILLING
596 mutant 8476. All were heterozygous and showed the laxatum-characteristic phenotype
597 (Figure S5 M-Q).

598 **Phenotypic analysis**

599 F2 plants and F3 progeny of recombinant plants were visually inspected for (i) width of the
600 lemma awn base, (ii) exposure of the mature caryopsis as well as (iii) number of anthers
601 after heading. Average rachis internode length was calculated by dividing overall ear length
602 of mature spikes by number of nodes per spike at full maturity (Figure S1).

603 **Preparation of genomic DNA**

604 Plant material for DNA isolation was harvested from greenhouse grown plants at three-leaf
605 stage. For genetic mapping a rapid 96-well plate format DNA isolation on the „Biorobot 3000“
606 (Qiagen, Hilden, Germany) system with MagAttract 96 DNA Plant Core Kit was performed
607 according to the manual (Qiagen, Hilden, Germany). Genomic DNA for exome capture was
608 isolated using a modified CTAB method (Stein et al., 2001). Genotyping of TILLING families
609 was performed by DNA extraction according to a modified CTAB protocol of Doyle (Doyle,
610 1990). Volumes of reagents were adjusted to 1.2 ml to accommodate to a 96-well format with
611 Collection Microtubes (Qiagen, Hilden, Germany).

612 **PCR amplification**

613 PCR was performed on GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, USA).
614 A standard touchdown PCR profile was used for all PCR analyses containing two cycling
615 steps: initial denaturation for 15 min at 95°C, followed by ten cycles of denaturation at 95°C
616 / 30 sec, annealing at 60°C / 30 sec (decreasing by 0.5°C per cycle) followed by extension at
617 72°C / 60 s); then 35 cycles denaturation at 95°C / 30 sec, annealing at 55°C / 30sec, and
618 extension at 72°C / 60 sec followed by a final extension step at 72°C / 7 min. PCR products

were resolved in 1.5% (w/v) agarose (Invitrogen GmbH, Darmstadt, Germany) gel by electrophoresis.

Genotyping and map construction

Marker development for genetic mapping of *HvLAX-A* followed a two-stage procedure. Initially, publicly available marker resources based on the previously defined ~30 cM introgressed segment for NIL BW457 (Druka et al., 2011) were evaluated. Since BW457 was crossed again with their recurrent parent cv. Bowman we could directly utilize polymorphic SNPs for mapping by using underlying sequence information (Close et al., 2009) to convert the array based marker into PCR based Cleaved Amplified Polymorphic Sequence (CAPS) marker (Konieczny and Ausubel, 1993) for low resolution mapping (Table S12). Restriction digests were performed according to manufacturer guidelines in a thermo cycler. SNPs used for low resolution mapping were converted to an 8-plex SNaPshot marker assay for screening an extended mapping population. Extension oligos are differentiating in sizes between 30 and 74 bp (Table S13). First, amplification of 92 to 227 bp fragments was performed by a multiplex PCR (two reactions, Table S13) using standard PCR conditions (see above). PCR reaction cleanup (removal of unincorporated dNTPs) was achieved by incubation with shrimp alkaline phosphatase and exonuclease 1 (Affymetrix, Santa Clara, USA). Reaction conditions as well as subsequent steps of sample preparation were performed according to the supplier`s protocol of the ABI PRISM SNaPshot™ Multipex Kit (Applied Biosystems, Foster City, CA, USA). Capillary electrophoresis was performed on Applied Biosystems 3730/3730xl DNA Analyzer equipped with 50cm capillaries, POP-7™ Polymer matrix and Data Collection Software 3.0 (Applied Biosystems, Foster City, CA, USA). The system was calibrated with Matrix Standard Set DS-02 (Set E5) according user bulletin (Applied Biosystems, Foster City, CA, USA). Peak histogram analysis for genotyping was done with GenMapper4.0 (Applied Biosystems, Foster City, CA, USA) software. Second source for marker development was based on known SNPs delivered from WGS survey sequencing of BW457 (see below). JoinMap version 4.0 (Kyazma B.V., Wageningen, The Netherlands) was used with the Kosambi mapping function to construct a linkage map.

Sanger sequencing

PCR amplicons were purified with NucleoFast 96 ultra-filtration plates (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Original PCR primers were used for sequencing using BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA) on the 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, USA). PHRED

20 quality (Ewing and Green, 1998; Ewing et al., 1998) trimmed sequences were analyzed with “Sequencher 5.2.3” software (Genecodes Corporation, USA).

Whole Genome Shotgun sequencing

Sequencing library preparation was performed according to standard protocols (IBSC, 2012). BW457 was sequenced to 8-fold coverage using the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA). Sequence reads were mapped against the Bowman WGS assembly (IBSC, 2012) with BWA version 0.5.9 (Li and Durbin, 2009). SNP calling was performed with SAMtools version 0.1.17 (Li, 2011) using default parameters.

Exome sequencing

DNA was quantified with Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and genotypes with shared marker haplotype / phenotype combination were pooled to equal amounts before sequence library preparation. One microgram of DNA was fragmented to a range of 200 – 400 bp by ultrasound using the Covaris S220 device (Covaris inc., Woburn, MA, USA) with the following settings: 175W Peak Incident Power, Duty Factor 10%, 200 cycles per burst and 100 seconds Treatment Time. Sample preparation was done with Illumina TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) based on the manufacturer’s instructions. Different samples or pools were individually indexed and captured together by a single liquid array capture assay (Mascher et al., 2013a; Himmelbach, 2014). Sequence of recombinants pools were mapped against the whole genome shotgun assembly of barley cultivar ‘Bowman’ with BWA 0.5.9 (Li and Durbin, 2009) using the parameter “-q 15” for quality trimming. Multi-sample SNP calling was performed with SAMtools 0.1.19 (Li, 2011) using the command “mpileup –q 10 –C50”. SNP calls were filtered for coverage and genotype score with a custom script adapted from ProtonGBSPaper (Mascher et al., 2013b). Read depth in capture targets was calculated with BEDtools (Quinlan and Hall, 2010). Genotype calls and coverage values were loaded into the R statistical environment and queried for segregation patterns identical to *lax-a* with custom scripts.

BAC sequencing

We sequenced selected, physically overlapping BAC clones that were representing the complete extension of the BAC contig (FPC_2862) on the Illumina MiSeq system (Table S6). Multiplexed library preparation sequencing on the Illumina HiSeq2000 (Illumina, San Diego, CA, USA) were performed based on published protocols (Beier et al., in press).

Physical anchoring and deletion detection

Physical anchoring was performed by PCR-based screening of two independent gene fragments of *HvLAX-A* (HvLAX_F/R1 and HvLAX_F/R3; Table S11) of multidimensional BAC pools (customized arrangement by Amplicon Express, Pullman, WA, USA) of the library HVVMRXALLeA (Schulte et al., 2011). *In silico* anchoring was done by manual inspection of BLASTN hits (Altschul et al., 1990) of sequenced BACs against physically anchored barley WGS contigs (IBSC, 2012).

The WGS data produced for mutant NIL BW457 (see above) was used to predict the size of the deletion. The fact that the sequenced BAC clones were generated from cultivar Morex required for a less stringent read mapping which allowed a small percentage of mismatches. In consequence of the short read length (2x100 bp paired end sequencing) an overrepresentation of highly similar short sequence reads is expected by mapping an entire genome against a short BAC contig, which would most likely lead to distorted read coverage. Therefore, we used the available 1.8 Gbp sequence assembly of genotype Bowman (IBSC, 2012) as a reference for mapping. The average read coverage for each Bowman WGS contig was calculated for the respective mapping of mutant and wild-type reads. Sixty-four sequence contigs of this WGS assembly of cv. Bowman, representing a cumulative length of 211 kbp, were assigned along the sequenced FPcontig by BLAST (alignment length ≥ 500; identity ≥ 99.5%). 17 of these WGS contigs, with a cumulative length of 53 kb, showed no sequence coverage by reads from the WGS assembly of the mutant NIL BW457 but the expected coverage of ~30-fold from the wild-type Bowman reads. All of these contigs were located in the central part of the sequenced FPcontig_2862 and were used to determine the deletion size (Figure S4).

Haplotype analysis

In order to study natural genetic diversity, the coding sequence was amplified from 303 genotypes (see Table S10) using three primer combinations for *HvLAX-A* (HvLAX_F/R4, HvLAX_F/R5, HvLAX_F/R6; Table S11) and *HvCUL4* (HvCUL4_F/R1, HvCUL4_F/R2, HvCUL4_F/R3; Table S15). All fragments were verified using a forward and reverse Sanger sequencing reaction. A haplotype analysis was performed based on re-sequencing data of the complete ORFs. Sequence alignments were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). DnaSP software package (Librado and Rozas, 2009) was used for haplotype detection under consideration of gaps. DNA ALIGNMENT 1.3.1.1 (http://www.fluxus-engineering.com) was used to produce the .rtf input file for the NETWORK 4.6.1.2 software (http://www.fluxus-engineering.com) to generate a Median-Joining network (Bandelt et al., 1999).

Phylogenetic analysis

Protein sequence homology search of HvLAX-A was performed by BLASTP (Altschul et al., 1990) against the barley high- and low-confidence gene set (IBSC, 2012) to identify paralogous genes in barley. To identify all *BOP-like* members within the plant kingdom, the NCBI protein (http://www.ncbi.nlm.nih.gov) and Phytozome v10.2 (http://phytozome.jgi.doe.gov) database were surveyed. For testing the presence of conserved protein domains in identified candidate protein sequences a conserved sequence search was performed at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple protein sequence alignment was performed using MUSCLE (Edgar, 2004a; Edgar 2004b). The phylogenetic analysis was performed using MEGA6 software (Tamura et al., 2013) following the published protocol (Hall, 2013). The Maximum Likelihood (ML) tree was constructed using the JTT model with discrete Gamma distribution and Nearest Neighbor Interchange (NNI) by applying 1000 Bootstrap replicates.

Data access

Illumina exome sequencing data of *lax-a* pools have been deposited at EMBL-ENA as accessions ERS463935 to ERS463939 (exome capture), assembled BAC sequences as accessions LO018452 to LO018472 and WGS raw data and sequence assembly of BW457 is available under accession ERS140281. Gene reference sequences from Sanger sequencing are available at EMBL-ENA (*HvLAX-A*: LN897709 and *HvCUL4*: LN897710).

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AUTHOR CONTRIBUTIONS

N.S and M.J. conceived the project. M.J. performed experiments with contributions of S.Taketa, T.Y. and F.S. M.Mascher analyzed targeted re-sequencing data. S.Taudien and A.H. performed NGS sequencing. B.S. and T.S. performed WGS sequence assembly; S.B. performed BAC assembly; U.S. contributed analysis tools. A.D. contributed mapping populations. M.J. and N.S wrote the article with contributions of R.W, S.Taketa, and M.Mascher. S.Taketa, M.Morgante, R.W. and N.S. designed the research. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1: *Laxatum-a* phenotype of BW457

A) The *laxatum* phenotype (*lax-a*) is characterized by an increased spike length caused by 15% longer rachis internodes compared to wild-type (Figure S1). B) Lodicules of wild-type plants are homeotically converted C) into additional stamenoid organs in lax-a mutants. D) Cross section through young wild-type (upper panel) and *lax-a* (lower panel) florets, respectively, showed that the additional stamen are smaller in size and comprising only two instead of four locules. E) compared to wild-type lemma and palea the F) *lax-a* type palea and lemma are much more narrow which G) leads to exposed and visible caryopses in mature *lax-a* inflorescences (right sample) and causes opening of flowers H) in *lax-a* mutants (right) while wild-type flowers stay closed I) Awns of *lax-a* plants (right) have a very wide base compared to wild-type (left).

Figure 2: Cloning-by-Sequencing of the gene *HvLAX-A*.

A sequence assisted evaluation of a predefined mapping interval was used to identify candidate genes underlying the *lax-a* phenotype. A) Eight recombinant plants out of 1,970 F2 plants delimited a 0.2 cM mapping interval on chromosome 5H. Marker scores and phenotype scores for each recombinant are represented by a color code for simplification (yellow = wild type, green = heterozygote, red = mutant). Genotypes with identical marker haplotype / phenotype combination were pooled for target enrichment re-sequencing (Mascher et al., 2013a). B) Obtained sequence reads of the individual pools were mapped to the reference of cv. Bowman (IBSC, 2012) for SNP discovery and determining SNP frequencies. Visualization of SNP frequency plots was restricted to chromosome 5H for each individual pool. The x-axis shows the physical expansion of 5H (IBSC, 2012), the y-axis represent the percentage of mapped reads with alternative mutant allele from 0 to 100% for each SNP (visualized as a dot) C) Filtering for candidates identified 27 targets on WGS sequence contigs. D) Identified High confidence (HC) and Low confidence (LC) genes (IBSC, 2012) on WGS contigs were used for homology analysis and revealed that seven of eight

identified putative homolog Brachypodium gene models cluster in a small syntenic interval of Brachypodium.

Figure 3: Schematic map of induced mutations in the gene *HvLAX-A*.

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Figure 4: Diversity analysis of *HvLAX-A* and *HvCUL4*.

Median-joining network derived from haplotypes identified by re-sequencing the ORF of (A) *HvLAX-A* and (B) *HvCUL4* in 83 *Hordeum spontaneum* accessions (yellow), 55 barley landraces (red), 150 barley cultivars (green), 17 accessions of breeding / research material (purple). Haplotypes were labeled with haplotype ID and number of accessions sharing the respective haplotype (brackets). Circle sizes are proportional to numbers of accessions per haplotype. Length of connector lines refers to number of nucleotide substitutions between haplotypes (indicated by numbers on connecting lines = number of mutations).

TABLES

Table 1: Physical map positions of genetically mapped markers

Marker ID	Barley high confidence gene[1]	Bowman WGS contig ID[1]	Genetic position[1] [cM]	Physical map contig[1]	Physical position[1] [Mbp]
1_0974	AK248898.1	contig_201113	17.92	FPcontig_1748	11,244,320
1_0580	MLOC_68535.2	contig_844634	31.25	n.a.	19,657,720
1_1198	MLOC_3013.1	contig_12040	43.95	n.a.	70,569,640
1_0157	AK251398.1	contig_884009	42.23	n.a.	153,040,800
1_1469	AK358200	contig_895954	42.23	FPcontig_44251	199,813,600
1_0481	MLOC_22626.1	contig_143316	42.23	n.a.	253,551,480
2_0524	AK356301	contig_221162	42.23	n.a.	248,503,440
HvLAX-A	MLOC_61451.6	contig_68343[2]	42.23	FPcontig_2862	203,930,400
Bd4g43680	MLOC_73193.3	contig_68744	42.23	FPcontig_3522	259,218,280
1_1260	MLOC_4554.1	contig_881005	44.51	n.a.	283,803,760
2_0239	MLOC_63089.10	contig_10702	42.23	n.a.	264,640,800
2_1148	MLOC_64817.1	contig_1786585[3]	45.9	n.a.	298,526,280
2_0713	MLOC_68788.2	contig_268627	51.45	n.a.	386,809,320

[1](IBSC, 2012); [2]contig was anchored by PCR based BAC-library screening; [3]Barke WGS; n.a. = not anchored to physical map

Table 2: Annotated genes located on FPcontig_2862:

HC_genes[1]	Annotation[1]	Brachypodium ID[2]
MLOC_61451.6	NPR1 protein	Bradi4g43150.1
AK373675	Strictosidine synthase family protein	Bradi4g43137.1
MLOC_69804.2	2-isopropylmalate synthase B, putative	Bradi4g43130.1
MLOC_10658.1	Cytochrome P450	Bradi4g43110.1

[1](IBSC, 2012), [2]Homologous genes in Brachypodium predicted by sequence similarity (BLAST)

Table 3: Resequencing independent *lax-a* accessions

NGB[1] Nr.	Mutant	Mutant type	Orig. variety	Mutagen used
116334	*lax-a.01*	complete deletion	Bonus	gamma-rays
116338	*lax-a.4*	C1983T (S348F)	Bonus	Cumarine
116342	*lax-a.8*	complete deletion	Bonus	Neutrons
116354	*lax-a.20*	complete deletion	Bonus	Neutrons
116372	*lax-a.37*	complete deletion	Bonus	Neutrons
116374	*lax-a.39*	complete deletion	Bonus	X-rays
116388	*lax-a.54*	T149C (F50S)	Bonus	ethylene imine
116426	*lax-a.92*	complete deletion	Bonus	X-rays
116436	*lax-a.208*	complete deletion	Foma	X-rays
116446	*lax-a.218*	A347T (Y116F)	Foma	ethylene imine
116450	*lax-a.222*	T125A (V42E)	Foma	ethylene imine
116458	*lax-a.229*	C26A (S9*)	Foma	Glyidol
116483	*lax-a.256*	A1505T (K194*)	Foma	iso-propyl methanesulfonate
116503	*lax-a.278*	C127T (L43F)	Foma	ethyl methanesulfonate (EMS)
116510	*lax-a.286*	C1853T (Q305*)	Foma	ethyl methanesulfonate (EMS)
116560	*lax-a.353*	complete deletion	Kristina	Neutrons
116579	*lax-a.369*	T512A (altered splicing)	Kristina	ethylene imine
116608	*lax-a.398*	T461G (L154R) 3 bp deletion (461; 462; 465)	Kristina	gamma-rays
116613	*lax-a.405*	complete deletion	Kristina	X-rays
116614	*lax-a.406*	complete deletion	Kristina	Neutrons
116622	*lax-a.413*	T123A (C41*)	Bonus	ethyl methanesulfonate (EMS)
116647	*lax-a.434*	complete deletion	Bonus	Neutrons
116650	*lax-a.437*	T1992A (L331H)	Bonus	iso-propyl methanesulfonate
116659	*lax-a.444*	complete deletion	Bonus	Neutrons
116664	*lax-a.448*	C184T (Q62*)	Bonus	ethyl methanesulfonate (EMS)
116668	*lax-a.450*	G417A (W139*)	Bonus	sodium azide
116675	*lax-a.455*	G417A (W139*)	Bonus	sodium azide
116695	*lax-a.472*	complete deletion	Bonus	X-rays
119823	*Naked caryopsis lax Erectoides 2*	complete deletion	Bonus	Neutrons

[1] Nordic genome resource center accession number

Table 4: Statistics of the diversity analysis of *HvLAX-A* and *HvCUL4*

	HvLAX-A			*HvCUL4*		
Parameter	**Wild**	**Dom.[1]**	**All**	**wild**	**Dom.[1]**	**all**
Number of haplotypes	26	13	31	10	2	10
Haplotype diversity (hd)	0.9412	0.765	0.8582	0.6644	0.0781	0.307
Number of sites	1548	1545	1551	1476	1476	1476
Number of sites[2]	1542	1542	1542	1476	1476	1476
Nucleotide diversity (π)	0.00456	0.00493	0.00499	0.0009	0.00005	0.00034
Polymorphic sites (S)	44	27	48	11	1	11
synonymous	34	24	38	9	1	9
non-synonymous	10	3	10	2	/	2
Tajima's D	-0.70735	1.72692	-0.03471	-1.06982	-0.53007	-1.61662

[1]Domesticated; [2]gaps excluded

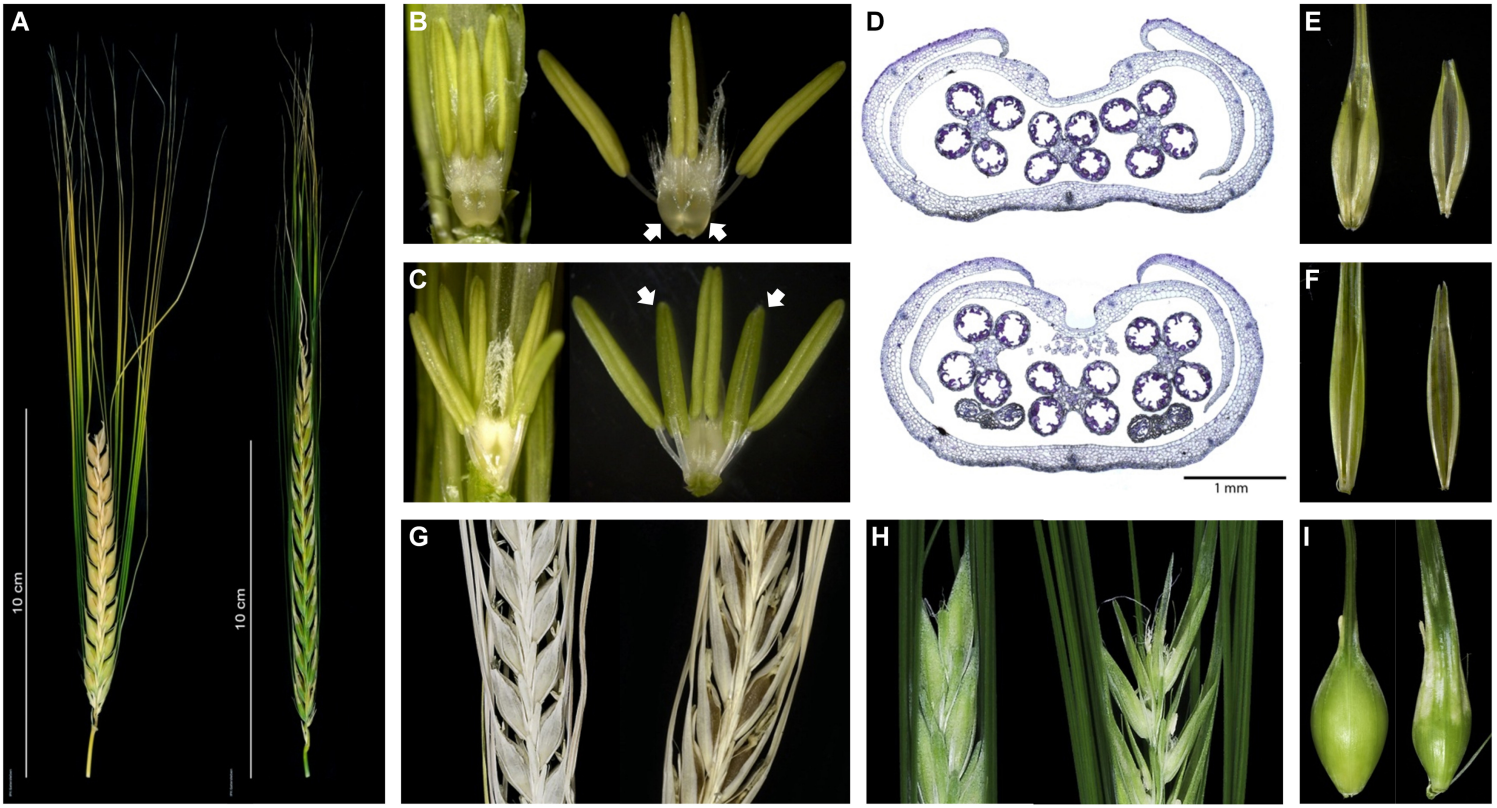
SUPPLEMENTAL DATA

Supplemental Figures:

Figure S1: Rachis internode length in *lax-a* mutant and wild type plants
Figure S2: Genetic mapping
Figure S3: Exome-capture SNP-Plots
Figure S4: Sequence analysis of FPcontig_2862 containing the *HvLAX-A* candidate gene
Figure S5: Independent lax-a mutant alleles and allelism test
Figure S6: BOP/ANK gene family of Arabidopsis and barley.
Figure S7: Phenotype of mutants obtained by TILLING of *HvCUL4*
Figure S8: Phylogenetic analysis of BOP-like genes in the plant kingdom

Supplemental Tables:

Table S1: Statistics exome-capture sequencing
Table S2: Filter for cosegregating targets with expected SNP frequency within captured pools
Table S3: Filtered candidate targets of read coverage analysis within captured pools
Table S4: Exome capture targets with SNPs which cosegregate with *HvLAX-A* phenotype
Table S5: Targets with low coverage in captured pools with mutant phenotype
Table S6: Sequenced BACs
Table S7: Genes on sequenced BACs of FPC_2862
Table S8: Identified TILLING mutants within *HvLAX-A*
Table S9: Identified TILLING mutants for *HvCUL4*
Table S10: Information of plant material used to identify sequence haplotypes of *HvLAX-A* and *HvCUL4*
Table S11: Oligonucleotides of *HvLAX-A*
Table S12: Marker information of genetic mapping
Table S13: SNaPshot genotyping assays
Table S14: SNP marker used for F1 test
Table S15: Oligonucleotides of *HvCUL4*



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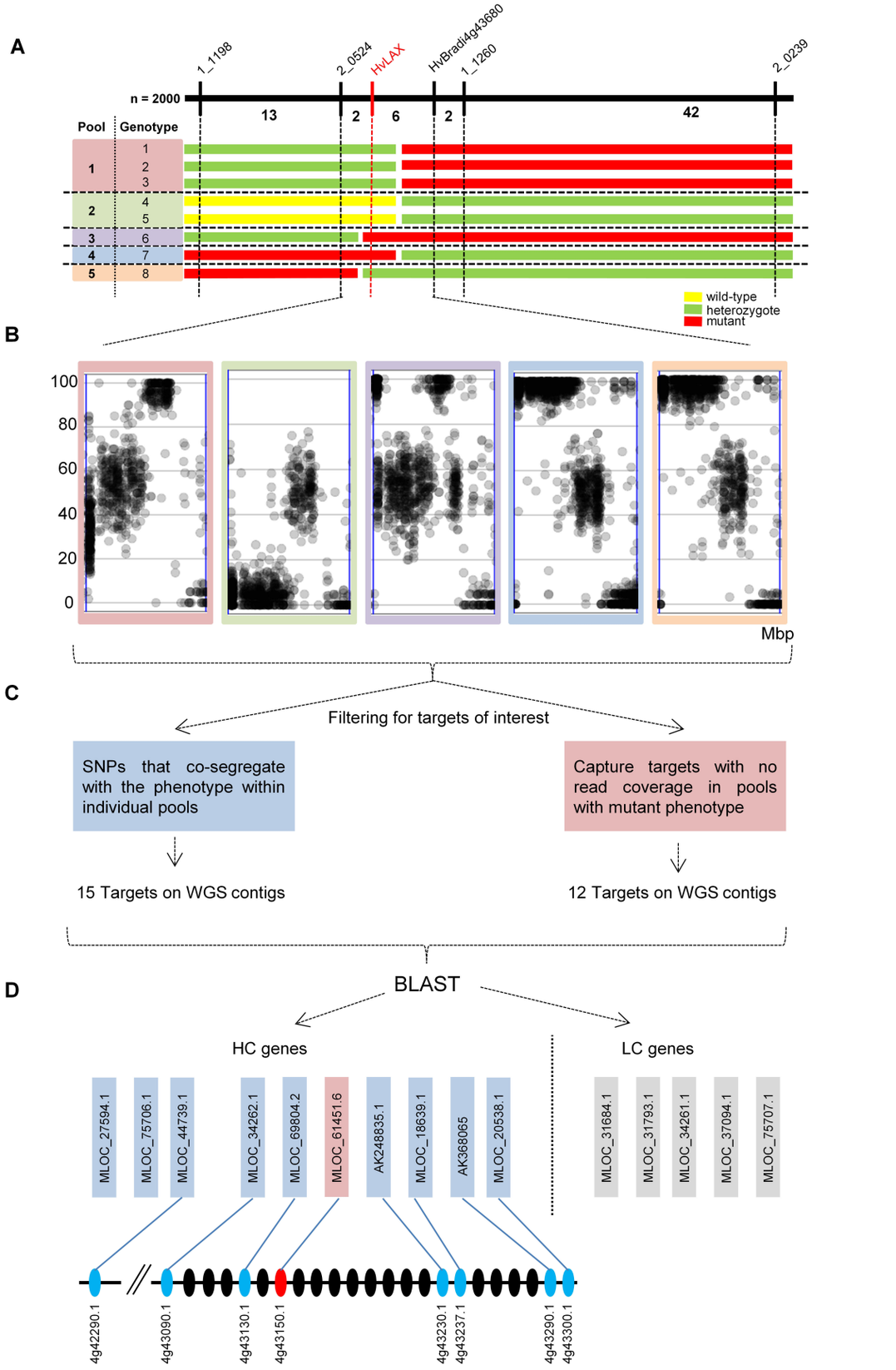

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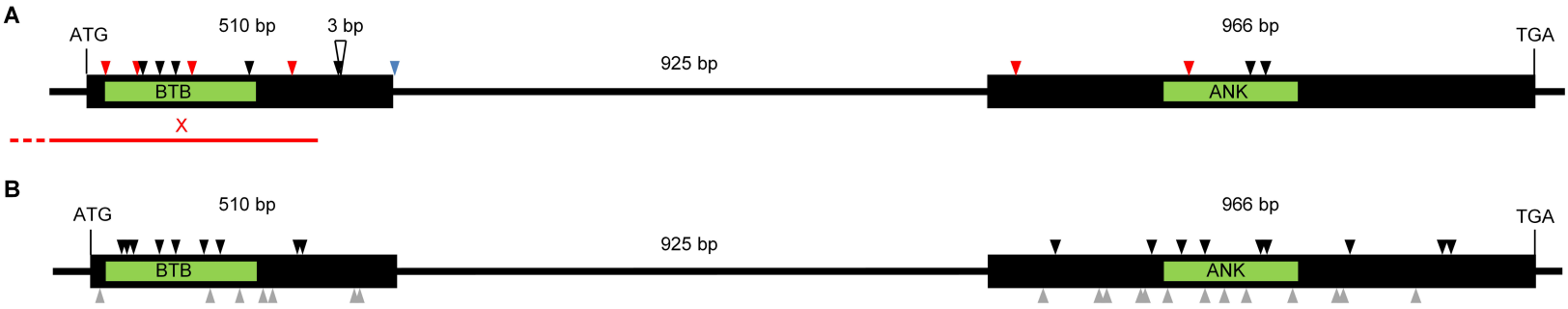

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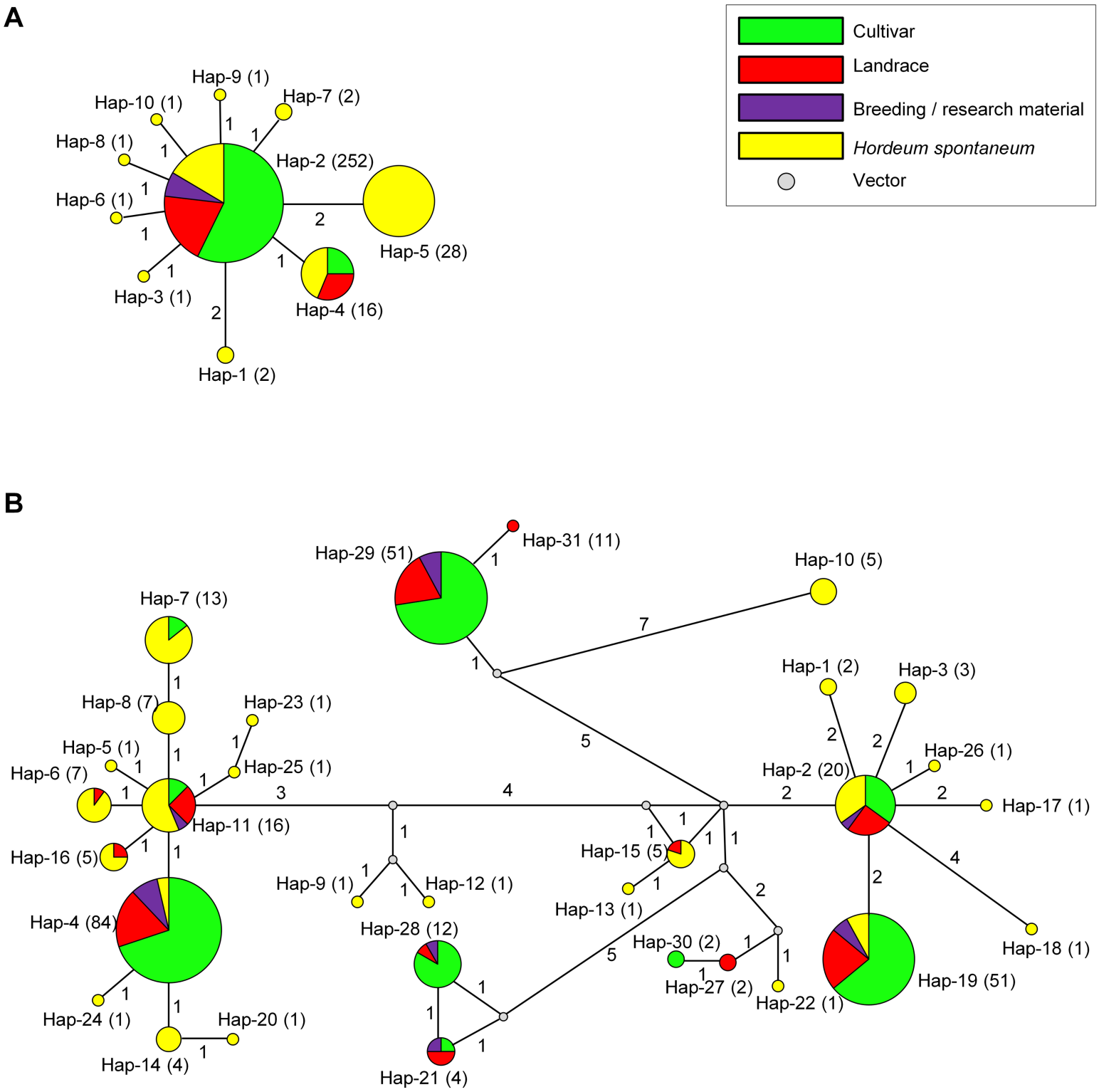

Figure 4: Diversity analysis of *HvLAX-A* and *HvCUL4*.

Median-joining network derived from haplotypes identified by re-sequencing the ORF of (A) *HvLAX-A* and (B) *HvCUL4* in 83 *Hordeum spontaneum* accessions (yellow), 55 barley landraces (red), 150 barley cultivars (green), 17 accessions of breeding / research material (purple). Haplotypes were labeled with haplotype ID and number of accessions sharing the respective haplotype (brackets). Circle sizes are proportional to numbers of accessions per haplotype. Length of connector lines refers to number of nucleotide substitutions between haplotypes (indicated by numbers on connecting lines = number of mutations).

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